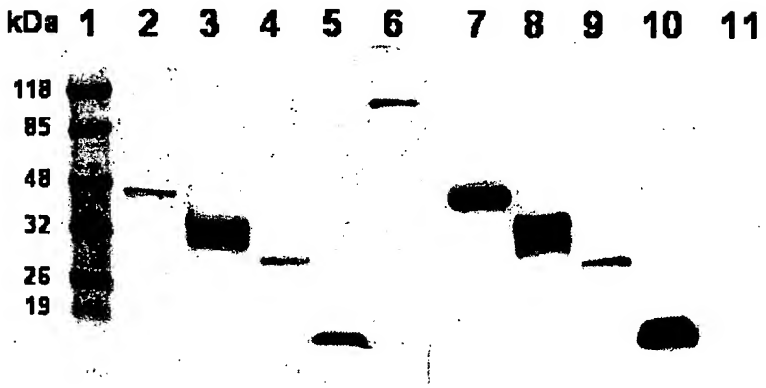
APPENDIX

Actin Binding Assay *in vitro*

Figure 1: Membrane Blotting

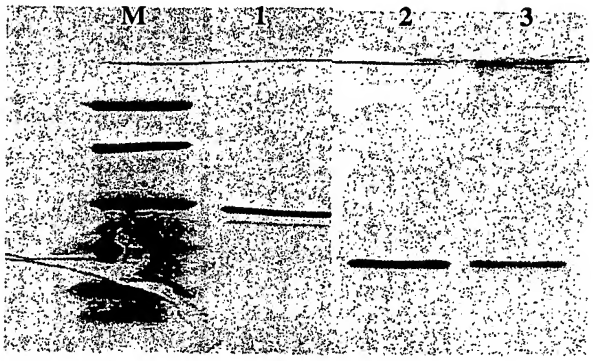


ACTIN BINDING OF T2 RNase

SDS-PAGE analysis of *A. niger* RNase T2 (ACTIBIND), angiogenin, *E. coli* RNase I, Actin (positive control) and BSA (negative control). The proteins were blotted on a nitrocellulose membrane and actin binding capability was accomplished following overnight incubation with 5 µg/ml G-actin. Proteins bound to actin were detected subsequent to reaction with anti-actin mAb followed by HRP-conjugated secondary antibody. Signals were detected by Super-Signal® enhanced-chemiluminescence system.

Lanes 1-6: SDS-PAGE protein staining. **Lanes 7-11**: Western blot analysis.
Lane 1: Molecular weight markers. **Lanes 2, 7**: actin; **Lanes 3, 8**: *A. niger* RNase T2 (ACTIBIND); **Lanes 4, 9:** *E. coli* RNase I; **Lanes 5, 10**: Angiogenin; **Lanes 6, 11**: BSA.

Figure 2: SDS-PAGE



SDS-PAGE analysis of Human RNase 6PL (Human T2 RNase) and its complex with G-Actin
M = SDS-Markers **Lane 1**: actin **Lane 2**: Human RNase 6PL **Lane 3**: 6PL+G-Actin Complex.

CONCLUSION: The western blot analysis (Figure 1) using G-actin to bind to different proteins demonstrated that *A. niger* T2 RNase, similar to angiogenin and *E. coli* RNase I, binds actin in a specific manner. Figure 2 demonstrates the avidity of actin binding by *A. niger* T2 RNase.

Figure 3: Pollen Tube Assay

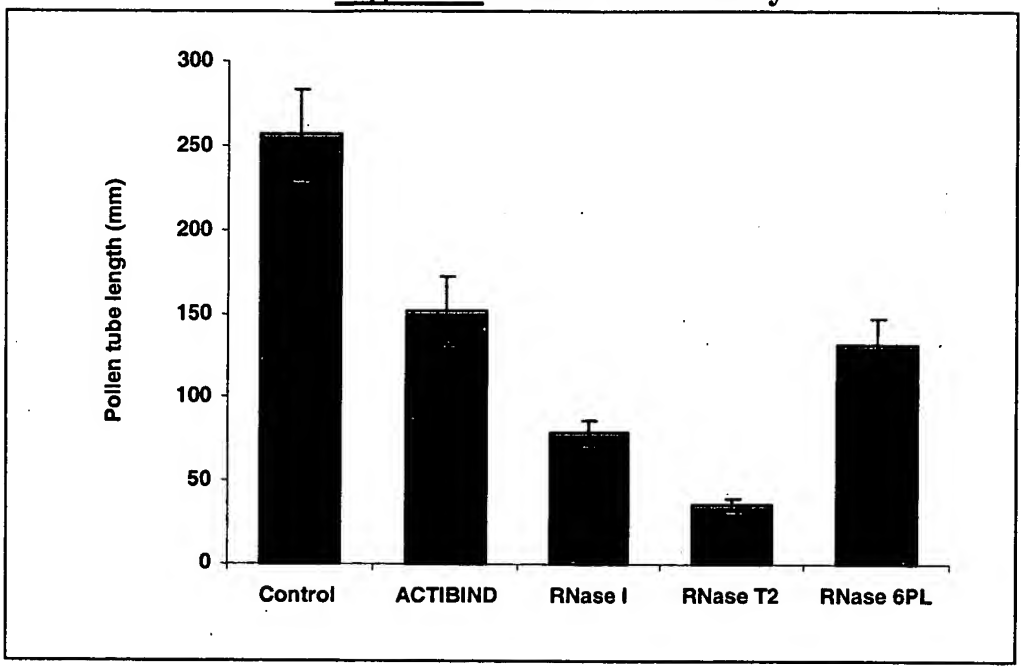


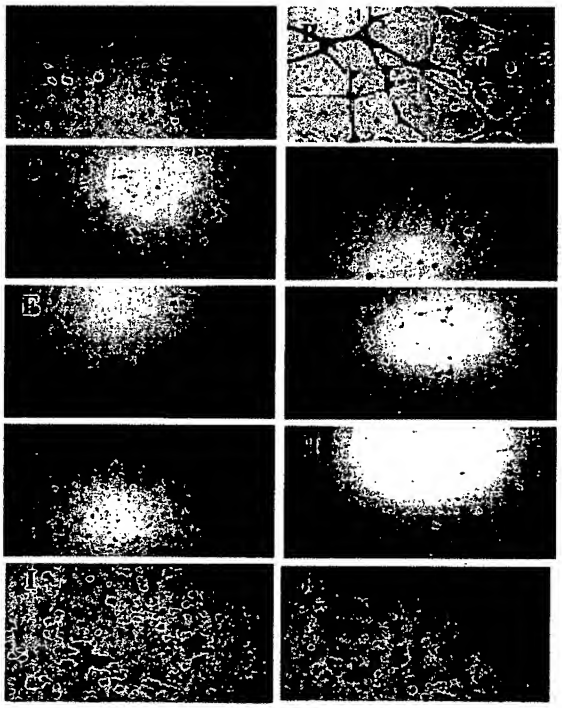
TABLE 1: Percent inhibition of pollen tube length in the presence of diverse RNases.

RNases	Control	ACTIBIND	RNase I	RNase T2	RNase 6PL
% Inhibition	0	40.9	69.1	86	48.8

Lily (*Lilium longiflorum* Thunb. cv. Osnat) flowers were maintained for 24 h at room temperature. Dehiscent anthers were then excised and pollen grains were germinated *in vitro* in 100 µl aqueous cultures containing 7% sucrose (w/v), 1.27 mM $Ca(NO_3)_2$, 0.16 mM H_3BO_3, 1 mM KNO_3 and 3 mM KH_2PO_4. The pollen cultures were supplemented with each of the different RNases tested to a final protein concentration of 1 µM. Following incubation (1.5 hours, 25°C) in the dark, pollen tube length was measured microscopically employing an eyepiece micrometer.

CONCLUSION: *A. niger* RNase T2 (ACTIBIND) similar to *E. coli* RNaseI, *A. oryzae* RNase T2 and RNase 6PL, the human T2 RNase, binds to endothelial surface actin in pollen tubes and has the ability to inhibit the growth and orientation of tip growing cells, and disrupts the intracellular actin network, leading to inhibition of cell protrusion formation and arrested cell motility.

Figure 4: HUVEC (Human Umbilical Vein Endothelial Cell) Angiogenesis Assay



T2 RNase of diverse origin inhibits angiogenesis in human cells:
Figure 4 summarizes the results as observed following the overnight incubation of HUVEC (human umbilical vein endothelial cells) tube formation on Matrigel in the presence or absence of 1μg/ml angiogenin, and in the presence or absence of different RNases (10μM each).

A. No angiogenin and no RNase (Control).

B. Angiogenin (Positive Control).

C. *A. niger* T2 RNase (ACTIBIND) (Negative Control)

D. *A. niger* T2 RNase (ACTIBIND) + angiogenin.

E. *A. oryzae*RNase T2 (Negative Control).

F. *A. oryzae* RNase T2 + angiogenin.

G. *E.coli* RNase I (Negative Control).

H. *E.coli* RNase I + angiogenin.

I. Human 6PL (Negative Control).

J. Human 6PL (Human T2 RNase) + angiogenin.

HUVE cells were plated at a density of 14,000 cells/well in a 96-well plate coated with growth factor-depleted Matrigel™ in M199 medium containing 5% FCS and 0.005% ECGF, supplemented with angiogenin or bFGF or recombinant VEGF to a final concentration of 1 μg/ml each. Different RNases (10 μM final concentration), or phosphate-buffered saline (PBS) were added. After overnight incubation, the plates were photographed and the extent of tube formation assessed. Six individual determinations were performed for each treatment.

CONCLUSION: HUVECs incubated in medium in the absence of angiogenin and RNase (Control) formed only few delicate tubes on the Matrigel surface (Fig.4A), whereas in the presence of angiogenin (Positive Control) massive tube growth is apparent (Fig. 4B). *A. niger* RNase T2 (ACTIBIND) or other T2-RNases of diverse origin had no effect on the cells when administered alone (Negative Control) (Fig.4C,E,G,I). However T2 RNases clearly inhibited angiogenin-induced tube formation (Fig.4D,F,H,J). A similar effect was observed in the presence of the other known angiogenic growth factors bFGF and VEGF (data not shown).

Figure 5: Clonogenicity Assay - Colony-Formation Assay

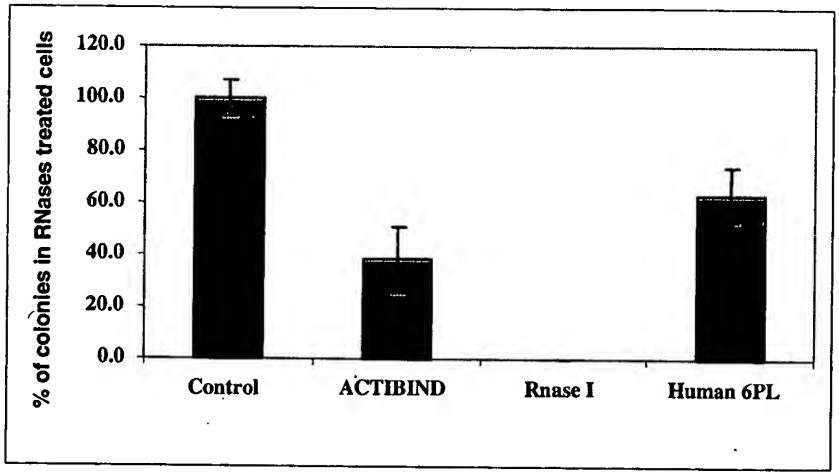


Figure 5

TABLE 2: Percent of colonies in T2 RNase treated cells.

RNases	Control	ACTIBIND	RNase I	RNase 6PL
% Colonies	100	36.3	0	58.3

T2 RNase of diverse origin effectively inhibits proliferation of Human Colon Cancer cells:

Figure 5 summarize the clonogenicity assay. The effect of *A. niger* T2 RNase (ACTIBIND) and other T2-RNases of diverse origin on *in vitro* cell cultures were tested. The colony-formation assay was performed with human colon cancer HT-29 cells. The cells were grown in 50-ml flasks at a concentration of 10^5 cells per flask. The medium contained 7 ml of DMEM supplemented with 10% FCS, 1% glutamine, and 1% antibiotic-antimycotic solution in the presence or absence of 1 μM *A. niger* T2 RNase (ACTIBIND), *E. coli* RNase I or RNase 6PL (Human T2 RNase). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 48 h, 1000 cells/well were seeded in 96-well plates in 200 μl medium, in the presence or absence of 1 μM of each RNase. After 5 days the colonies were counted. Six individual determinations were performed for each RNase.

CONCLUSIONS: In the presence of all T2-RNases, regardless of origin, the number of Human Colon Cancer colonies was significantly lower than in the control.

Figure 6: *in vivo* Assay - Xenografts Assay.

Figure 6A

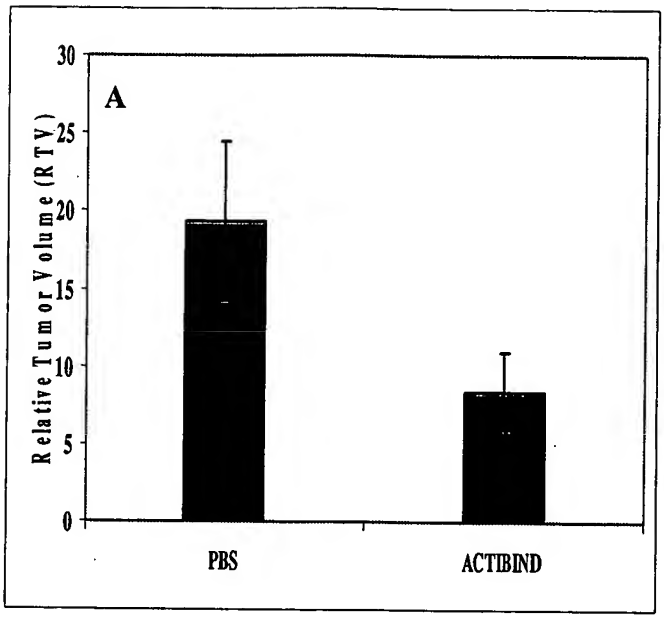
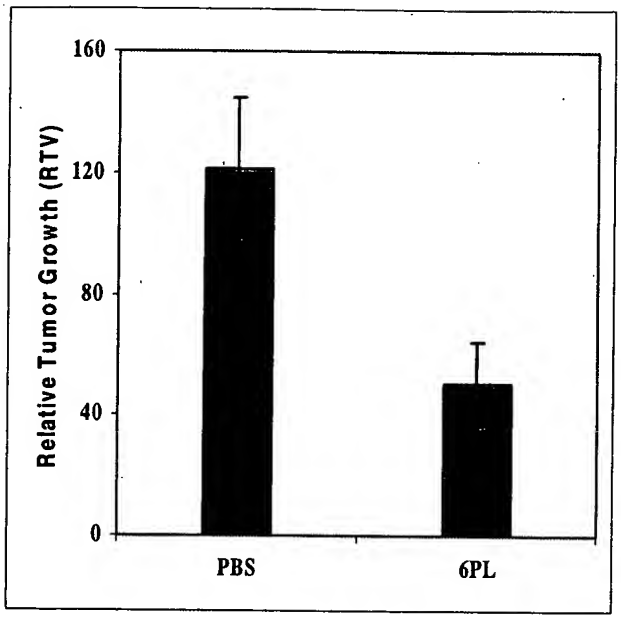


Figure 6B



RNases	Control	ACTIBIND	RNase 6PL
% of Relative Tumor Volume	100	43.7	41.7

T2 RNase of diverse origin effectively inhibits Human Colon Cancer tumor growth in mice:

Viable human colon cancer LST174 cells (500,000/100 µl per mouse) were injected subcutaneously into the left hip of athymic mice (CD-1 nu/nu; Charles River, Wilmington, MA). *A. niger* T2 RNase (ACTIBIND) (Fig.6A), RNase 6PL (Human T2 RNase) (Fig.6B) or PBS were injected intraperitoneally every two days (0.5 mg/kg/100 µl of each RNAse). Mice were examined daily for tumor appearance and development. After 30 to 42 days the mice were sacrificed, and the tumors or area of injection were harvested, weighed and processed for histopathologic and immunostaining examinations. The experiments were repeated twice, using 10 mice per treatment.

Relative Tumor Volume (RTV): V_i / V_0, where V_i is the tumor volume at any given time and V_0 is that at the time of initial treatment (Fujii T et al. Cancer Research (2003), 23: 2405-2412).

CONCLUSIONS: Administration of fungal (*A. niger*) or Human (RNase 6PL) T_2-RNases, regardless of origin, effectively inhibited Human Colon Cancer tumorogenicity in the mouse xenograft assay.